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Quantitative measurement of salivary testosterone in Korean adults by stable isotope-dilution liquid chromatography-electrospray-tandem mass spectrometry

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Salivary testosterone levels in Korean adults were quantitatively measured for the first time by liquid chromatography-electrospray-tandem mass spectrometry (LC ESI MS/MS). Salivary testosterone was separated on a multiple reaction monitoring (MRM) chromatogram within 7 min. The LC ESI MS/MS assay was validated over the linearity range of 0.01-2.00 ng/ml (r=0.99987) using testosterone-d_3 as an internal standard. The lower limit of quantification (LOQ) was 0.01 ng/ml. The intra- and inter-assay precisions were 1.54% to 4.09% and 0.96% to 4.29%, respectively. The mean recovery was 93.32% (range 88.43-98.05%). The validated assay was then applied to measure the salivary testosterone levels of Korean adults. In men, the salivary testosterone level collected between 9 : 00-11 : 00 am was approximately 2.8 times higher than that in women ($P < 0.0001$). Salivary testosterone levels in both sexes negatively correlated with age. The present assay would also be useful in measuring salivary testosterone levels in clinical laboratories. [BMB reports 2010; 43(11): 761-765]

INTRODUCTION

Testosterone is produced by testicular Leydig cells in males and by the adrenal glands, ovaries, and peripheral conversion of circulating androstenedione in females (1). In men, testosterone is known to be necessary for the maintenance of spermatogenesis, secondary sexual characteristics, bone density, muscle mass, and libido (2). Serum testosterone concentration in women is known to be approximately 5-10% of that in men and is considered to be important in the maintenance of bone mineral density, mood, and libido (1, 3).

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Measurement of testosterone has many clinical applications and is essential in the evaluation of androgenic status and monitoring of stimulatory, suppressive, or replacement therapy in children and adults. Especially, testosterone in men is largely measured to evaluate late-onset hypogonadism (LOH) (4-7), whereas the steroid in women is measured to evaluate hyperandrogenism such as hirsutism, acne, alopecia, and oligo-amenorrhea (8, 9).

In both men and women, the majority of circulating testosterone is bound to proteins. In healthy adult men, about 98% of circulating testosterone is bound to serum proteins, primarily sex hormone-binding globulin (SHBG) and albumin, whereas only 1-2% of serum testosterone is free of bound protein (10). Albumin-bound testosterone and free testosterone are referred to as bioavailable testosterone, which is thought to be a good index of androgen activity (11). A significant correlation between salivary and serum free testosterone has been demonstrated in healthy subjects, indicating that salivary testosterone can be a good index of serum free testosterone (6, 12-14). Due to these reasons, salivary testosterone has recently attracted attention for use in the evaluation of physiological and pathological conditions based on steroid assays.

Among the steroid assays, immunoassay-based methods for salivary testosterone evaluation are known to be unsatisfactory due to cross-reactivity between a variety of endogenous materials, and the measured values often overestimate the true concentrations, especially at the low levels of testosterone typically found in women, children, men with androgen deficiencies, and patients undergoing anti-androgenic therapies (15-17). To circumvent these problems, LC MS/MS assay has been used as a highly specific and sensitive tool for evaluating salivary testosterone in healthy adults in Japan or USA, using protocols specified by the Federal Drug Administration (FDA) (4, 5, 18). However, to the best of our knowledge, no study reported the evaluation of salivary testosterone levels in Korean adults of both sexes by LC MS/MS assay.

The aims of the present study were to validate the LC ESI MS/MS method for measuring testosterone concentrations in saliva samples from both sexes and to quantify the salivary testosterone levels in Korean adults.

RESULTS AND DISCUSSION

Chromatographic separation

Salivary testosterone was eluted within 7 min by the gradient elution method without any interfering substances (Fig. 1B, C, D). No ion suppression was observed. The testosterone was clearly separated in the MRM chromatogram of an extracted saliva sample. The retention times of testosterone and testosterone-d_3 were 6.25 min and 6.22 min, respectively (Fig. 1C, D). This result indicates that the rapid analytical time would permit high-throughput measurement of salivary testosterone.

Validation performance

LC ESI MS/MS assay for measuring salivary testosterone levels was validated. The assay was linear from 0.01 to 2.00 ng/ml (Fig. 1A). The regression coefficient (r) of the calibration curve ($y = 0.62189\ x + 0.00469$) was 0.99987, indicating excellent linearity. The LOQ was 0.01 ng/ml (Table 1). In our study, the LOQ was low enough to quantitatively measure salivary testosterone (Fig. 1B-D). Intra- and inter-day (n=5) accuracies ranged from 92.40% to 97.72% and from 96.28% to 96.90%, respectively, whereas intra- and inter-day precisions (n=5) ranged from 1.54% to 4.09% and from 0.96% to 4.29%. The average recoveries of salivary testosterone in triplicate were 88.43% for 0.05 ng/ml spike, 93.48% for 0.50 ng/mL spike, and 98.05% for 2.00 ng/ml spike (Table 1). These results indicate that the present analytical method was within internationally accepted criteria and was fast, highly reproducible, accurate, specific, and sensitive.

Salivary testosterone measurements from healthy adults in Korea

The average concentrations of salivary testosterone were 69.36 ± 17.95 pg/ml (range: 38.50-99.50 pg/ml) for men and 25.52 ± 9.80 pg/ml (10.10-46.00 pg/ml) for women between 9 : 00-11 : 00 a.m. At the collection time, the salivary testosterone level in men was approximately 2.8 times higher than that in women ($P < 0.0001$) (Fig. 2A and Table 2). It is well known that salivary testosterone in human is subject to significant diurnal rhythms and individual differences. Further, inter-day alterations in steroid levels in the early morning (6 : 00-7 : 00 a.m.) are larger than in the late morning (9 : 00-11 : 00 a.m.), as evidenced by LC ESI MS/MS assay (4, 5). To avoid the individual differences and inter-day alterations in salivary testosterone level in the early morning, we analyzed saliva samples collected between 9 : 00-11 : 00. As pre-

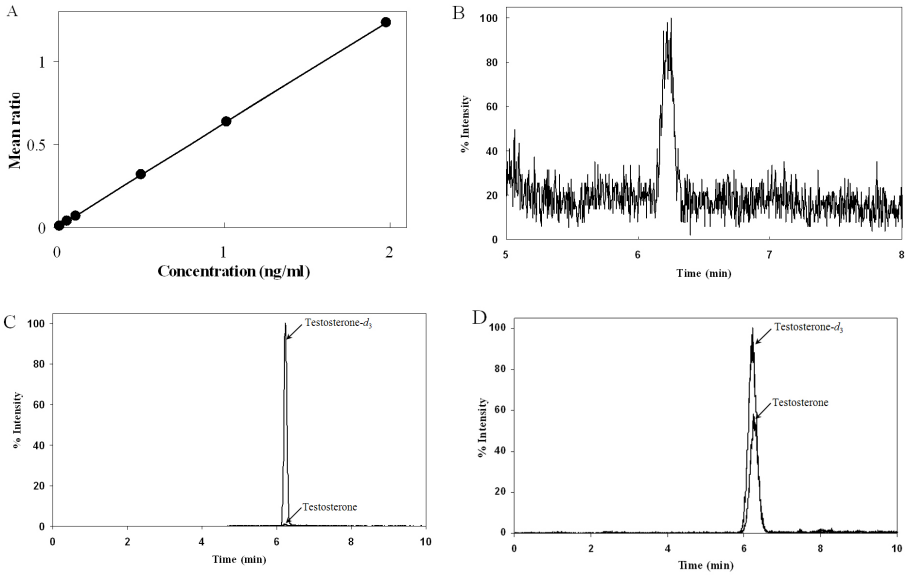

Fig. 1. (A) Calibration curve at spiked concentrations of 0.01 to 2.00 ng/ml for testosterone from steroid-free saliva. (B) MRM chromatogram of an extracted saliva sample spiked with testosterone at LOQ concentration of 0.01 ng/ml. (C) MRM chromatogram of an extracted saliva sample (1 ml) containing 0.01 ng of testosterone and a 100 µl aliquot of testosterone-d_3 solution with a concentration of 100 ng/ml. (D) MRM chromatogram of an extracted saliva sample (1 ml) containing 1.00 ng of testosterone and a 100 µl aliquot of testosterone-d_3 solution with a concentration of 100 ng/ml.

Table 1. Intra- and inter-day accuracy, precision, recovery, and LOQ of the LC ESI MS/MS assay for measuring salivary testosterone

Concentration (ng/ml)	Intra-assay (n = 5)		Inter-assay (n = 5)		Recovery (%)	LOQ (ng/ml)
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)		
0.05	97.72	4.09	96.28	4.29	88.43	0.01
0.50	92.40	2.10	96.32	0.96	93.48	
2.00	92.90	1.54	96.90	2.38	98.05	

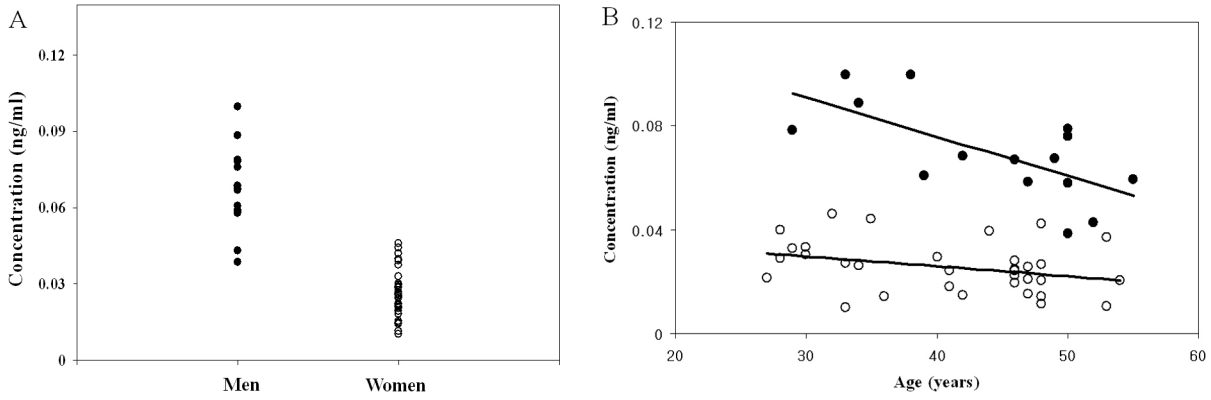

Fig. 2. (A) Salivary testosterone levels in men (●) and women (○) Korean adults (n = 48) collected between 9 : 00-11 : 00 a.m. (B) Relationship of salivary testosterone with age in men (●) and women (○). Regression equations were $y = -0.0015\ x + 0.1364$ for men and $y = -0.0004\ x + 0.0411$ for women.

Table 2. Concentrations (pg/ml) of salivary testosterone between 9 : 00 −11 : 00 a.m

	Concentration ± SD[a] (range)
Men	69.36 ± 17.95 (99.50-38.50)
Women	25.52 ± 9.80 (46.00-10.10)

[a]SD: standard deviation

viously reported by LC MS/MS assay, mean levels of salivary testosterone were found to be 40.22 pg/ml (n = 51, range = 30-85 years old) between 9 : 00-9 : 30 a.m. (12), or 40.00 to 90.00 pg/ml (n = 22, range = 20-65 years old) between 9 : 00-17 : 00 (4) in Japanese healthy men. Therefore, the salivary testosterone levels in Korean healthy men were within a reasonable range compared with those in Japanese healthy men. Salivary testosterone levels measured by the present LC ESI MS/MS assay also declined with age in both sexes (Fig. 2B). Salivary testosterone levels in men negatively correlated with age ($R = -0.44$, $P < 0.05$), whereas the testosterone levels in women showed a tendency toward negative correlation, although the relation was not significant ($R = -0.10$, $P = 0.28$) (Fig. 2B). Generally, it has been reported that salivary testosterone levels are inversely correlated with age (4-7, 19)

In this study, we evaluated the salivary testosterone levels in Korean adults using the validated LC ESI MS/MS assay method. Based on the data, the assay was fast, sensitive, specific, and reliable enough to enable high-throughput screening for salivary testosterone in clinical laboratories. Therefore, the present assay as a non-invasive approach is expected to be useful in the evaluation of androgen status and in the diagnosis of androgen disorders. Moreover, it could be applicable for monitoring salivary testosterone after replacement therapy in Korean adults.

MATERIALS AND METHODS

Chemicals and materials

Testosterone was purchased from TCI (Tokyo Kasei Kogyo Co., Japan). Deuterated testosterone (testosterone-d_3, 98%) as an internal standard was purchased from Sigma (St. Louis, MO, USA). All chemicals were of analytical grade, and all solvents including methanol, acetonitrile, and water were of HPLC grade. All solvents were filtered through Advantec® membranes (pore size 0.45 μm, Toyo Roshi Kaisha, Ltd., Japan) before use.

LC ESI MS/MS

High performance liquid chromatography (HPLC) was performed using an Agilent 1200 series (Palo Alto, CA, USA) equipped with an auto-injector. All separations were achieved on a Capcellpak UG 120 C8 (2.0 mm x 100 mm, 5 μm, Shiseido, Japan). The initial mobile phase was composed of water and methanol (40 : 60, v/v). Salivary testosterone was separated by gradient elution at a flow rate of 0.2 ml/min. The initial mobile phase was gradually changed to 70% methanol for 0.5 min, 95% methanol for 2.5 min, and 100% methanol for 1 min, after which elution with 100% methanol was maintained for 1 min. The gradient was then returned to the initial conditions (water-methanol (40 : 60, v/v)) for 1 min and held for 6 min before injecting the next sample. Sample injection volume was 5 μl. Column temperature was kept at 20°C.

The levels of salivary testosterone were measured using a triple-quadruple mass spectrometer (API-4000™) equipped with a TurboIonSpray® probe (ABI/MDS Sciex, CA, USA). The following *m/z* MRM transitions were selected: 289.17 → 109.10 for testosterone and 292.19 → 109.20 for testosterone-d_3. The instrument was operated in positive-ion mode with an ionspray potential (IS) of 5500 V and source temperature of 450°C. For testosterone, declustering potential (DP) of 81 V, entrance potential (EP) of 10 V, collision cell exit potential

(CXP) of 8 V, and collision energy (CE) of 35 eV were used as instrumental parameters. For testosterone-d_3, DP of 81 V, EP of 10 V, EXP of 10 V, and CE of 33 eV were used as parameters. Data were acquired and processed with Analyst™ 1.5 software. The calibration curves were constructed using peak area ratios of testosterone to testosterone-d_3 of the calibration standards and by applying a weighted (1/X) linear regression.

Sample collection and sample preparation

Saliva from 15 male healthy volunteers (median age: 44.2 ± 7.9 years olds) and 33 female healthy volunteers (median age = 41.0 ± 8.2 years old) was collected into a commercial Salivette® tube (Sarstedt, Nümbrecht, Germany) between 09 : 00-11 : 00 am and stored at −70°C until use. All volunteers took no hormone supplements or drugs that affect androgen biosynthesis and metabolism. The volunteers did not consume food or beverage and did not brush their teeth within 1 h before the sample collection. Informed consent was obtained from all volunteers. The saliva tubes collected were centrifuged at 4,000 rpm for 5 min. The supernatants were stored at −70°C until analysis. Stock solutions of testosterone were prepared at concentrations of 100 μg/ml in 100% methanol and stored at 4°C. The stock solutions were used to spike calibration standards and quality control samples. Saliva pooled from healthy volunteers was stirred with 1 g of activated charcoal (Sigma, St. Louis, MO, USA) overnight and then centrifuged at 4,000 rpm for 15 min. The supernatant was used as the steroid-free saliva. Working calibrators were prepared by dilution of the stock solutions in the steroid-free saliva. Calibration standards and quality control samples were stored at 4°C until analysis.

Sample extraction

After thawing, the saliva samples (1 ml) were mixed with a 100 μl aliquot of the internal standard solution having a concentration of 100 ng/ml. The extraction was carried out using a Oasis® HLB 1 cc solid phase extraction (SPE) column (Waters, MA, USA). Each saliva sample spiked with the aliquot of the internal standard was passed through the SPE column, which was pre-equilibrated with 1 ml of methanol followed by 1 ml of water. After washing with 50% methanol, testosterone was finally eluted with 100% methanol. The eluates were evaporated to dryness under a gentle nitrogen stream and then reconstituted with 80% methanol. The calibration standards and quality control samples, which were both spiked with a 100 μl aliquot of the internal standard, were also extracted using the same method as above. The analytes were injected into LC MS/MS.

Method validation

All validation steps were performed following international guidelines (20). Linearity was assessed by the correlation coefficients (r^2) on the calibration curve constructed with six different concentrations ranging between 0.01 and 2.00 ng/ml. The LOQ with a signal-to-noise (S/N) ratio of 10 was defined as the lowest concentration of acceptable precision on the calibration curve in which the relative standard deviation (R.S.D.) was $<20\%$. Method precision and accuracy were determined from the QC samples at three different concentrations of testosterone (0.05, 0.50, and 2.00 ng/ml). Intra-assay accuracy and precision were evaluated by analyzing the three QC samples five times in 1 day. Inter-assay accuracy and precision were evaluated by analyzing the samples over five different days. Recoveries (%) were calculated by dividing the measured concentrations by the three different spiked concentrations (0.05, 0.50, and 2.00 ng/ml). To investigate possible interfering substances, pooled saliva spiked with 10 ng/ml of cortisol, cortisone, corticosterone, 11-deoxycortisol, progesterone, testosterone, 17-hydroxyprogesterone, dihydrotestosterone, aldosterone, and dehydroepiandrosterone were analyzed.

Statistical analysis

Salivary testosterone levels in men and women were evaluated statistically using Student *t*-test. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using GraphPad software (QuickCalcs, La Jolla, CA).

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REFERENCES

1. Burger, H. (2002) Androgen production in women. *Fertil. Steril.* **77(Suppl 4)**, 3-5.
2. Griffin, J. E. and Wilson, J. D. (1998) Disorders of the testes and the male reproductive tract; in *Williams Textbook of Endocrinology,* Wilson, J. D., Foster, D. W., Kronenberg, H. M. and Larsen, R. R. (eds.), 9th ed. pp. 819-875, Saunders Company, Philadelphia, USA.
3. Synder, P. (2001) Editorial: The role of androgens in women. *J. Clin. Endocrinol. Metab.* **86**, 1006-1007.
4. Matsui, F., Koh, E., Yamamoto, K., Sugimoto, K., Sin, H., Maeda, Y., Honma, S. and Namiki, M. (2009) Liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay for simultaneous measurement of salivary testosterone and cortisol in healthy men for utilization in the diagnosis of late-onset hypogonadism in males. *Endocr. J.* **56**, 1083-1093.
5. Shibayamaa, Y., Higashia, T., Shimadaa, K., Odania, A., Mizokamib, A., Konakab, H., Kohb, E. and Namikib, M. (2009) Simultaneous determination of salivary testosterone and dehydroepiandrosterone using LC-MS/MS: Method development and evaluation of applicability for diagnosis and medication for late-onset hypogonadism. *J. Chromatogr. B.* **877**, 2615-2623.
6. John, E., Morley, J. E., Perry III, H. M., Patrick, P., Dollbaum, C. M. and Kells, J. M. (2006) Validation of salivary testosterone as a screening test for male hypogonadism. *Aging Male* **9**, 165-169.
7. Goncharov, N., Katsya, G., Dobracheva, A., Nizhnik, A.,

Kolesnikova, G., Herbst, V. and Westermann, J. (2006) Diagnostic significance of free salivary testosterone measurement using a direct luminescence immunoassay in healthy men and in patients with disorders of androgenic status. *Aging Male* **9**, 111-122.
8. Karrer-Voegeli, S., Rey, F., Reymond, M. J., Meuwly, J. Y., Gaillard, R. C. and Gomez, F. (2009) Androgen dependence of hirsutism, acne, and alopecia in women: retrospective analysis of 228 patients investigated for hyperandrogenism. *Medicine (Baltimore)* **88**, 32-45.
9. Swinkels, L. M., van Hoof, H. J., Ross, H. A., Smals, A. G. and Benraad, T. J. (1991) Concentrations of salivary testosterone and plasma total, non-sex-hormone-binding globulin-bound, and free testosterone in normal and hirsute women during administration of dexamethasone/synthetic corticotropin. *Clin. Chem.* **37**, 180-185.
10. Dunn, J. F, Nisula, B. C. and Rodbard, D. (1981) Transport of steroid hormones: binding of 21 endogenous steroids to both eestosterone-binding globulin and corticosteroid-binding globulin in human plasma. *J. Clin. Endocrinol. Metab.* **53**, 58-68.
11. Morley, J. E. (2007) The diagnosis of late life hypogonadism. *Aging Male* **10**, 217-220.
12. Yasuda, M., Honma, S., Furuya, K., Yoshii, T., Kamiyama, Y., Ide, H., Muto, S. and Horie, S. (2008) Diagnostic significance of salivary testosterone measurement revisited: using liquid chromatography/mass spectrometry and enzyme-linked immunosorbent assay. *J. Mens Health* **5**, 56-63.
13. Arregger, A. L., Contreras, L. N., Tumilasci, O. R., Aquilano, D. R. and Cardoso, E. M. L. (2007) Salivary testosterone: a reliable approach to the diagnosis of male hypogonadism. *Clin. Endocrinol.* **67**, 656-662.
14. Shirtcliff, E. A., Granger, D. A. and Likos, A. (2002) Gender differences in the validity of testosterone measured in saliva by immunoassay. *Horm. Behav.* **42**, 62-69.
15. Taieb, J., Mathian, B., Millot, F., Patricot, M., Mathieu, E., Queyrel, N., Lacroix, I., Somma-Delpero, C. and Boudou, P. (2003) Testosterone measured by 10 immunoassays and by isotope-dilution gas chromatography-mass spectrometry in sera from 116 men, women, and children. *Clin. Chem.* **49**, 1381-1395.
16. Herold, D. and Fitzgerald, R. (2003) Immunoassays for testosterone in women: better than guessing? *Clin. Chem.* **49**, 1250-1251.
17. Wang, C., Catlin, D. H., Demers L. M., Starcevic, B. and Swerdloff, R. S. (2004) Measurement of total serum testosterone in adult men: comparison of current laboratory methods versus liquid chromatography-tandem mass spectrometry. *J. Clin. Endocrinol. Metab.* **89**, 534-543.
18. Kushnir, M. M, Takara Blamires, T., Rockwood, A. L., Roberts, W. L., Yue, B., Erdogan, E., Bunker, A. M. and Meikle, A. W. (2010) Liquid chromatography-tandem mass spectrometry assay for androstenedione, dehydroepiandrosterone, and testosterone with pediatric and adult reference intervals. *Clin. Chem.* **56**, 1138-1147.
19. Ellison, P. T., Bribiescas, R. G., Bentley, G. R., Campbell, B. C., Lipson, S. F., Panter-Brick, C. and Hill, K. (2002) Population variation in age-related decline in male salivary testoterone. *Hum. Reprod.* **17**, 3251-3253.
20. Guidance for Industry: Bioanalytical Method Validation. (2001) Department of Health and Human Services. F.D.A., USA.